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Effect of Third Component on Water Structure around Tryptophan in Aqueous Solution[1,2]

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The effect of 3rd component (additive) on the water structure around tryptophan molecule was discussed on the basis of solubility properties of tryptophan in aqueous urea solution and of the adsorption of tryptophan by carbon black from aqueous EtOH solution.

Considering that the iceberg formation around non-polar molecules is an enthalpy effect at room temperature on the basis of Shinoda and Fujihira's new view on the hydrophobic bonding,[9] the following mechanism seemed reasonable for the increase in solubility of tryptophan upon addition of urea: urea comes in contact with hydrophobic moiety of tryptophan to result in a simultaneous structural change (a kind of destruction) of the iceberg around that moiety, accompanying an increase of its affinity to water to result in a predominantly large decrease in enthalpy of mixing.

The decrease in adsorption of tryptophan from aqueous solution with the addition of EtOH also might be due to the contact between EtOH and the carbon black surface or the tryptophan molecule containing a simultaneous structural change of iceberg, in the same way as described above.

Higher solubility of L-tryptophan than DL-isomer was considered to come from its stronger molecular interaction, as was suggested by a differential scanning calorimetry.

Solubility properties of tryptophan in 1, 3-dimethyl urea solution and EtOH solution suggested that the effects of these additive on the hydrophilic moiety were not negligible.

Various discussions have been given as to solubility properties of hydrocarbons in aqueous media and the effect of urea on the iceberg formation, such as the increase of solubility of alkane (except methane) in water caused by the entropy increase through the destruction of iceberg upon addition of urea,[4] the participation of urea in the formation of urea-water cluster,[5] or the partition of alkane in both dense and lattice parts in water where urea exists in the dense part only.[6] Unlike currently accepted views on the hydrophobic bonding,[7,8] Shinoda and Fujihira concluded,[9] as a coherent explanation by the extention of the regular solution theory, that the enthalpy of mixing of non-polar solute with water is large, but the enthalpy decrease due to the iceberg formation of water largely cancels the enthalpy increase of mixing and thus the apparent enthalpy of solution is small or negative at low temperature, also concluding that the small solubility of non-polar solute in water is not an entropy effect, and the solubility of non-polar solute is promoted by the iceberg formation of water molecules.

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1) This paper forms part VI of "Physico-chemical Approach to Biopharmaceutical Phenomena." Preceding paper, Part V: H. Nogami, T. Nagai, and H. Uchida, *Chem. Pharm. Bull.* (Tokyo), **17**, 176 (1969).
2) A part of this work was presented at the 89th Annual Meeting of the Pharmaceutical Society of Japan, Nagoya, April 1969.
3) Location: *Hongo, Tokyo*; *a*) To whom communications should be directed.
4) D.B. Wetlaufer, S.K. Malik, L. Stoller, and R.L. Coffin, *J. Am. Chem. Soc.*, **86**, 508 (1964).
5) M. Abu-Hamdiyyah, *J. Phys. Chem.*, **69**, 2720 (1965).
6) H.S. Frank and F. Franks, *J. Chem. Phys.*, **48**, 4746 (1968).
7) W. Kauzman, *Advances in Protein Chem.*, **14**, 1 (1959).
8) G. Nemethy and H.A. Scheraga, *J. Chem. Phys.*, **36**, 3401 (1962).
9) K. Shinoda and M. Fujihira, *Bull. Chem. Soc. Japan*, **41**, 2612 (1968).

In the previous papers,[10-13] the existing view on hydrophobic bonding seemed reasonable to explain the solubility properties of tryptophan and the adsorptions of tryptophan and barbituric acid derivatives by carbon black under the experimental conditions. However, as will be described later, there are found difficulties in explaining the above phenomena under various conditions by the same view, while a much more reasonable explanation is given on the basis of the new view by Shinoda and Fujihira.[9]

The present study was attempted to investigate thermodynamically the effect of urea on the partial molar volume of hydrophobic moiety of tryptophan in water. Considering that the iceberg formation is an enthalpy effect at room temperature,[9] the following mechanism seemed reasonable for the increase in solubility of tryptophan upon addition of urea: urea comes in contact with the hydrophobic moiety of tryptophan to result in a simultaneous structural change (a kind of destruction) of the iceberg around that moiety, accompanying an increase of its affinity to water. An additional investigation on the same view was made as to the adsorption of tryptophan by carbon black from aqueous EtOH solution in relation to the solubility. Such circumstances may extend to an approach to an understanding of the denaturation of globular protein in relation to the solubility of tryptophan upon addition of various substances, as will be discussed in the following paper.[14]

Experimental

Materials——L-Tryptophan, DL-tryptophan, MeOH, EtOH, urea, 1,3-dimethyl urea, and ethyleneurea of the purest reagent grade were obtained commercially. Carbon black used was the same as that in the previous paper.[10]

Procedure for the Determination of Solubility of Tryptophan——Excess of L- or DL-tryptophan (about 0.8 g) was added in 20 ml of aqueous solution, being stirred for 24 hr[15] in a constant temperature bath, and the mixture was filtered rapidly with a glass-filter.[16] The concentration of tryptophan in the filtrate was determined according to ultra violet (UV) absorption method at 279 mμ after diluting with distilled water, using a Hitachi Perkin-Elmer 139 UV-VS spectrophotometer.

Differential Scanning Calorimetry——A Perkin-Elmer differential scanning calorimeter DSC-1B was used at a scanning rate of 8°/min. Weights of samples were 1.08 mg and 1.19 mg for L- and DL-tryptophan, respectively.

Procedure for Determination of the Amount Adsorbed by Carbon Black——Putting 20 mg of carbon black in 10 ml of various aqueous solutions of tryptophan, the procedure was carried out in the same way as described in the previous paper.[10]

General Concepts

Following the concept that enthalpy and entropy of solution of non-polar solutes in water diverge strikingly from the normal behavior established for regular solutions and this abnormality is mostly due to the iceberg formation around solute molecules in water,[9] the entropy, $\Delta\bar{S}$ (enthalpy, $\Delta\bar{H}$) of dissolution of solid (2nd component) in water is expressed by combining the entropy of fusion, ΔS^f (enthalpy of fusion, ΔH^f) and the entropy due to the iceberg formation, ΔS^i (enthalpy due to the iceberg formation, ΔH^i), as follows:

$$\Delta\bar{S} = -R \ln X_2 + \Delta S_2{}^f + \Delta S_2{}^i \tag{1}$$

$$\Delta\bar{H} = V_2\phi_1{}^2 B' + \Delta H_2{}^f + \Delta H_2{}^i \tag{2}$$

$$B' = (\delta_1 - \delta_2)^2 \tag{3}$$

10) H. Nogami, T. Nagai, E. Fukuoka, and H. Uchida, *Chem. Pharm. Bull.* (Tokyo), **16**, 2248 (1968).
11) H. Nogami, T. Nagai and H. Uchida, *Chem. Pharm. Bull.* (Tokyo), **16**, 2257 (1968).
12) H. Nogami, T. Nagai, and H. Uchida, *Chem. Pharm. Bull.* (Tokyo), **16**, 2263 (1968).
13) H. Nogami, T. Nagai, and H. Uchida, *Chem. Pharm. Bull.* (Tokyo), **17**, 168 (1969).
14) H. Nogami, T. Nagai, and H. Umeyama, *Chem. Pharm. Bull.* (Tokyo), **18**, 335 (1970).
15) This was satisfactorily long to attain to equilibrium.
16) The adsorption of solute by the filter was preliminarily examined to be negligible.

where X_2 is the molar fraction of solid (2nd component), V_2 the molar volume, ϕ_1 the volume fraction of solvent, and B' the experimental constant depending on the solubility parameters δ_1 and δ_2 of solvent and solute, respectively. Sum of the first and the second terms corresponds to the respective function of solution in regular solution.

Upon discussion of the thermodynamic functions of dissolution of solid in an aqueous solution of 3rd component (additive), ΔS^f and ΔH^f may not be influenced by the additive at a constant temperature in the solution involving no special interaction between solute and additive. In the case of tryptophan, no complex formation has been recognized by the differential absorbance spectrophotometry in various solvents[17] and in aqueous solution containing ethylene urea.[18] It is, therefore, expected that any special interaction different from the water–tryptophan interaction does not take place between tryptophan and additive. Accordingly, the thermodynamic functions of transfer of tryptophan from its aqueous solution to aqueous solution of 3rd component are considered to be influenced by the respective functions of mixing and of structural change of iceberg. In other words, $\Delta\bar{S}_2$, $-R\ln X_2$, ΔS^i_2, $\Delta\bar{H}_2$, $V_2\phi_1^2B'$ and ΔH^i_2 vary with the addition of 3rd component. Here it is considered that the entropy change, $\Delta\bar{S}_{2/3}$ (enthalpy change, $\Delta\bar{H}_{2/3}$) caused by the addition of 3rd component is composed of entropy of mixing, $\Delta S^m_{2/3}$ (enthalpy of mixing, $\Delta H^m_{2/3}$) and entropy of structural change of iceberg, $\Delta S^i_{2/3}$ (enthalpy change of structural change of iceberg, $\Delta H^i_{2/3}$). Then, noting the thermodynamic functions of tryptophan in water as $\Delta\bar{S}_{2/0}$, $-R\ln X_{2/0}$, $\Delta S^i_{2/0}$, $\Delta\bar{H}_{2/0}$, $V_{2/0}\phi_1^2B'$, and $\Delta H^i_{2/0}$, the following equations are derived.

$$\Delta\bar{S}_{2/0}+\Delta\bar{S}_{2/3}=-R\ln X_{2/0}+\Delta S^f_{2/0}+\Delta S^i_{2/0}+\Delta S^m_{2/3}+\Delta S^i_{2/3} \tag{4}$$

$$\Delta\bar{H}_{2/0}+\Delta\bar{H}_{2/3}=V_{2/0}\phi_1^2B'+\Delta H^f_{2/0}+\Delta H^i_{2/0}+\Delta H^m_{2/3}+\Delta H^i_{2/3} \tag{5}$$

At equilibrium,

$$\Delta\bar{S}_{2/3}=\Delta\bar{H}_{2/3}/T=\Delta H^m_{2/3}/T+\Delta H^i_{2/3}/T.$$

Then, equation (4) is modified as

$$\Delta\bar{S}_{2/0}+\Delta H^m_{2/3}/\mathrm{T}+(\Delta H^i_{2/3}/T-\Delta S^i_{2/3}) = -R\ln X_{2/3}+\Delta S^f_{2/0}+\Delta S^i_{2/0} \tag{6}$$

Taking equation (6) from (1), the remainder corresponds to the free energy change of transfer of tryptophan from its aqueous solution to aqueous solution of 3 rd component, which is expressed as follows:

$$\Delta H^m_{2/3}/T+(\Delta H^i_{2/3}/T-\Delta S^i_{2/3})=-R\ln(X_{2/3}/X_{2/0}) \tag{7}$$

The right hand of equation (7) corresponds to $\Delta S^m_{2/3}$, being negative when the solubility of tryptophan increases upon addition of 3rd component. According to Shinoda and Fujihira's analysis,[9] the change, $(\Delta H^i_{2/3}/T-\Delta S^i_{2/3})$ is regarded to be an enthalpy predominant effect, as being positive when the iceberg is broken down. Therefore, to make the left hand of equation (7) negative, $\Delta H^m_{2/3}/T$ should overcome $(\Delta H^i_{2/3}/T-\Delta S^i_{2/3})$. Since V_2 and ϕ_1^2 are not considered to vary upon addition of 3rd component, $\Delta H^m_{2/3}$ may depend on the change of B', *i.e.*, δ_1 and δ_2.

Conclusively, the concept regarding the enthalpy of mixing described above will be useful to discuss the effect of 3rd component (additive) on the structure of iceberg around solute molecule in water.

17) K. Hamaguchi, K. Hayashi, T. Imoto and M. Funatsu, *J. Biochem.*, **55**, 24 (1964).
18) By the authors' preliminary examination, using a Hitachi 124 spectrophotometer.

Results and Discussion

Solubility Properties of L- and DL-Tryptophan in Aqueous Solution Containing 3rd Component

Difference in Solubility between L- and DL-Tryptophan

Fig. 1 and Table I show that the solubility of L-tryptophan is higher than that of DL-tryptophan in any case. From the temperature dependences of solubilities in water obtained

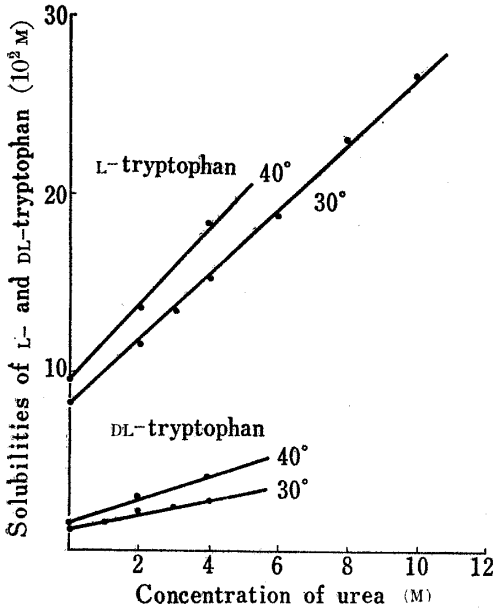

Fig. 1. Effect of Urea on Solubilities of L- and DL-Tryptophan

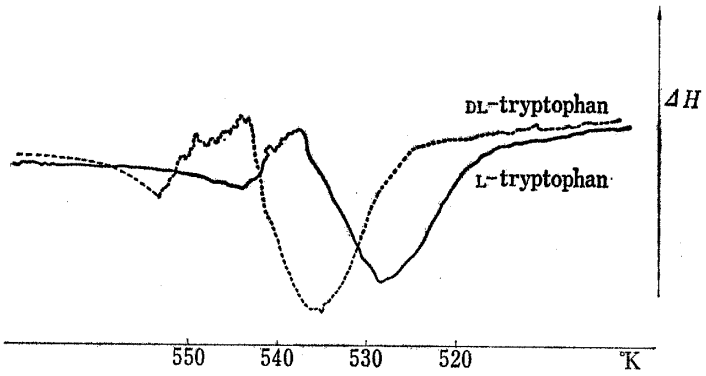

Fig. 2. Thermograms of Tryptophan by DSC at Scanning Speed of 8°/min

Table I. Solubibilities of L- and DL-Tryptophan in 2M Solutions of Addtives ($\times 10^2$M)

Additive	L-Tryptophan			DL-Tryptophan		
	30°	40°	50°	30°	40°	50°
Nothing	8.10	9.48	11.22	1.20	1.57	—
Urea	11.52	13.50	15.68	2.07	2.83	—
1,3-Dimethylurea	12.10	14.36	—	2.05	2.52	—
Ethyleneurea	16.26	18.16	—	2.74	3.40	—
MeOH	6.60	8.33	—	—	—	—
EtOH	6.18	8.45	—	—	—	—

at 30°, 40°, and 50°: $\Delta\bar{H}_2$(L)=3.13 kcal/mol; $\Delta\bar{H}_2$(DL)=4.87 kcal/mol, and then from equation (1): ΔS_2^f(L)+ΔS_2^i(L)=−2.65 e.u. and ΔS_2^f(DL)+ΔS_2^i(DL)=−0.70 e.u., where L and DL mean L- and DL-tryptophan, respectively. Here it is considered that ΔS_2^i does not vary with the optical isomers of tryptophan and also there is no difference in $V_2\phi_1{}^2B'$ because δ_2 is the same for the isomers. Therefore, the differences in $\Delta\bar{H}_2$ and in (ΔS_2^f+ΔS_2^i) between both isomers may come from those in ΔH_2^f and in ΔS_2^f, which may originally come from the difference in the molecular interaction in solid state. For this problem a differential scanning calorimetry gave a suggestive information, as shown in Fig. 2. The exothermic reactions in Fig. 2 are considered to correspond to the respective phase transitions of crystals, taking place arround 256° for L-tryptophan and around 272° for DL-tryptophan, and thus the mol ecular interaction of DL-tryptophan is considered to be stronger than that of L-tryptophan.

Effect of Urea on the Structural Change of the Iceberg around Tryptophan

Upon addition of urea, the respective solubilities of L- and DL- tryptophan increased in the similar way, as shown in Fig. 1, and the decreasing tendency in $-\ln(X_{2/3}/X_{2/0})$ also were common to both L- and DL- tryptophan, as shown in Table II. These results show that even the solution of L-isomer, which is more soluble and convenient enough to the experiment, can be treated as a dilute one.

Table II. $\Delta F_{2/3}/T = -R \ln X_{2/3}/X_{2/0}$ of the Transfer of Tryptophan from Aqueous Solution to Aqueous Urea Solution at 30° (cal/mole·degree)

Ureas	L-Tryptophan	DL-Tryptophan
1M Urea	−0.351	−0.592
2M Urea	−0.700	−1.067
3M Urea	−0.946	−1.319
4M Urea	−1.300	−1.609
2M 1,3-Dimethylurea	−0.798	−1.043
2M Ethyleneurea	−1.384	−1.620

From the concept described already, $(\Delta H^{i}_{2/3}/T - \Delta S^{i}_{2/3})$ in equation (7) becomes positive when the iceberg around tryptophan molecule is broken down by urea, and thus $\Delta H^{m}_{2/3}$ should overcome $(\Delta H^{i}_{2/3}/T - \Delta S^{i}_{2/3})$ and the solubility parameters δ_1 and δ_2 should be taken into consideration. For discussion of the solubility parameter of tryptophan, δ_2 is assumed to consist of $\delta_2(a)$ and $\delta_2(b)$ which are the solubility parameters for the hydrophobic and the hydrophilic moieties, respectively. The solubility parameter of water at 25° is 23.8,[19] while that of hydrophobic molecule is generally less than 10. Considering that solubility parameter depends on aggregation energy of molecule, it may be assumed that an ion has a larger solubility parameter than water. Therefore, it is considered possible that $\delta_2(a) < \delta_1 < \delta_2(b)$ in water. Nozaki and Tanford reported that the free energy change of transfer of glycine from aqueous solution to aqueous urea solution was 1 to 7% of that of tryptophan, the solubility of glycine decreasing with the addition of urea.[20] These facts show that urea gives effect on the hydrophobic moiety of tryptophan to make its solubility increase by the following mechanism: urea comes in contact with the hydrophobic moiety of tryptophan under an equilibrium constant at the respective concentration to result in a simultaneous structural change (a kind of destruction) of the iceberg around that moiety, accompanying an increase of its affinity to the solvent (*i.e.*, water). Here, the contact between tryptophan and urea is assumed to be almost independent of bonding energy between them, and the contact product may be in the state that tryptophan is surrounded by a decreased iceberg, so $\delta_2(a)$ being larger than that in the water without urea. Such an increase of $\delta_2(a)$ upon addition of urea is considered to result in decrease of B', that is, of the enthalpy change of mixing, and thus $\Delta H^{m}_{2/3}/T$ may overcome $(\Delta H^{i}_{2/3}/T - \Delta S^{i}_{2/3})$.

Solubilities of tryptophan in aqueous solutions containing urea derivatives are shown in Table I. The addition of ethyleneurea resulted in a greater increase of the solubility than the case of urea, while 1,3-dimethylurea made the solubility a little higher than urea. Anyhow, such derivatives gave greater effect on the solubility increase of tryptophan than urea. Kay and Evans reported that the increasing tendency for iceberg destruction was urea>methylurea>1,3-dimethylurea according to conductivity measurement.[21] Therefore, it seems difficult to explain the increase of solubility with the addition of urea derivatives on the basis

19) K. Shinoda, "Yoeki to Yokaido," Maruzen, Tokyo, 1966, p. 103.
20) Y. Nozaki and C. Tanford, *J. Biol. Chem.*, **238**, 4074 (1963).
21) R.L. Kay and D.F. Evans, *J. Phys. Chem.*, **70**, 2325 (1966).

of the simple destruction of iceberg or cluster, while it may be reasonable to explain the same phenomena on the basis of the contact between tryptophan and 3rd component to result in the simultaneous structural change of iceberg accompanying a large decrease in the enthalpy of mixing, as was described above.

Feldman and Gibaldi also reported that the increasing effect of alkyl ureas on the solubility of salicylic and benzoic acids was 1,3-dimethylurea>methylurea>urea.[22] Moreover, Robinson, *et al.*, reported that the solubilities of benzene, benzyl alcohol, and others increased more with the addition of 1,3-dimethylurea than with the addition of urea.[23] These results also may be explained on the concept regarding the enthalpy of mixing in the same way as described above.

A remarkable increase in the solubility of tryptophan upon addition of ethyleneurea shown in Table I seemed to be a much increased effect of ethylene group on the hydrophobic moiety of tryptophan, while, in the case of 1,3-dimethylurea, the effect of dimethyl groups on the hydrophilic moiety of tryptophan might be produced, giving the result that the solubility of tryptophan did not increase so much as the case upon addition of ethyleneurea. Similar discussions will be given in the following paper[14] in relation to the effects of various organic substances on the solubility properties of tryptophan in aqueous solution.

Effect of Ethyl Alcohol on the Adsorption of L-Tryptophan by Carbon Black

Adsorption isotherms of L-tryptophan by carbon black from aqueous EtOH solutions in Fig. 3, which are in accordance with Langmuir equation in the same way as described in the previous paper,[10] show that the adsorbed amount decreased with the addition of EtOH, though the degree of the effect of EtOH decreased with its concentration.

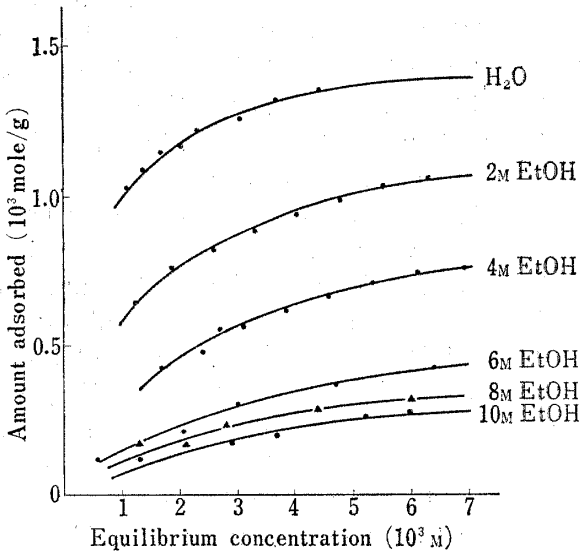

Fig. 3. Effect of EtOH on the Adsorption of L-Tryptophan by Carbon Black from Aqueous Solution at 30°

Generally, when the adsorption from solution is in accordance with Langmuir equation, the equilibrium constant of adsorption process depends on (1) the adsorption energy and (2) the rate constants of adsorption and desorption of solute on the adsorbent. The former varies with the interaction force between solute and adsorbent (*e.g.*, van der Waals force), but it may not be influenced by the additive in the case of the adsorption of tryptophan by carbon black from aqueous solution. Therefore, the effect of additive on the latter should be taken into consideration. If the adsorbent is hydrophobic like carbon black, the following equation is derived as an extension of equation (7),

$$\Delta H^m_{2/3}/T+(\Delta H^i_{2/3}/T-\Delta S^i_{2/3})+(\Delta H^b_{2/3}/T-\Delta S^b_{2/3}) = -R\ln(X_{2/3}/X_{2/0}) \quad (8)$$

where the suffix 2 means the adsorbent, $\Delta H^b_{2/3}$ ($\Delta S^b_{2/3}$) the enthalpy (entropy) due to the bonding between adsorbent and 3rd component (EtOH), and $X_{2/3}$ and $X_{2/0}$ express the partial molar affinity of adsorbent to water upon addition of 3rd component and without 3rd component, respectively. In the system of carbon black/EtOH, the bonding enthalpy is considered to

22) S. Feldman and M. Gibaldi, *J. Pharm. Sci.*, **56**, 370 (1967).
23) D.R. Robinson and W.P. Jencks, *J. Am. Chem. Soc.*, **87**, 2462 (1965).

be very small, because even DL-phenylalanine took a negative adsorption[12] and moreover the bonding entropy decreases upon contact. Therefore, the term $(\Delta H^{b}_{2/3}/T-\Delta S^{b}_{2/3})$ in equation (8) may be negligible in the present system, and thus the contact of EtOH with carbon black also may be discussed by equation (7) in a similar way to the case of solubility properties. In other words, the enthalpy of mixing, $\Delta H^{m}_{2/3}$ is considered to be a predominant factor.

In the previous papers,[10-13] the adsorptions of tryptophan and barbituric acid derivatives by carbon black were explained on the basis of the hydrophobic bonding mechanism in which the hydrophobic moiety of molecule played an important role, and the larger the hydrophobic moiety, the easier it was adsorbed bacause the larger iceberg might be destructed upon adsorption. Considering these facts, the dercease in adsorption with the addition of EtOH shown in Fig. 3 might be due to the contact between EtOH and the carbon black surface or between EtOH and the tryptophan molecule to result in a simultaneous structural change of iceberg, as was discussed already for the increase in solubility of tryptophan with the addition of urea.

Upon the contact of EtOH with carbon black mentioned above, the hydrophobicity of carbon black surface may get smaller than that in the plain water, lowering the escaping tendency of tryptophan from solution to carbon black surface to result in a decrease of adsorption. Since the carbon black surface is considered to be more hydrophobic than the hydrophobic moiety of tryptophan, the iceberg around the former may be more influenced by EtOH than that around tryptophan. In other words, the decrease in the enthalpy of mixing upon contact of EtOH with carbon black surface is considered to be much larger than that upon contact of EtOH with tryptophan, and thus the former may be predominantly effective for the decrease in the adsorption of tryptophan by carbon black. Additionally, it should be described that the present study is not concerned with the general competitive adsorption, which is taken into consideration when the respective adsorbates have a similar degree of adsorbability. Therefore, the difference in adsorbability on carbon black between EtOH and tryptophan is not discussed, but the effect of EtOH (3rd component) on the hydrophobicity of carbon black (2nd component) in water (1st component), which is evaluated by the adsorbability of tryptophan, is discussed from the thermodynamical viewpoint on the basis of the consideration that EtOH has a very low adsorbability on carbon black compared with tryptophan and the term due to bonding energy in equation (8) may be negligible.

It is shown in Table I that the solubility of tryptophan in 2M EtOH was smaller than that in water, while the adsorbed amount of tryptophan by carbon black from the same EtOH solution was not larger than that from the aqueous solution without EtOH, as shown in Fig. 3. Therefore, the adsorption can not be explained as a reverse process of dissolution, but may be explained by considering such a contact of EtOH with carbon black surface as described above. On the other hand, the decrease in solubility of tryptophan upon addition of EtOH comes from the predominant effect of EtOH on the hydrophilic moiety of tryptophan. In this connection, if the effect of additive on the hydrophilic moiety is negligible compared with that on the hydrophobic moiety under experimental conditions, the adsorption will apparently be observed as a reverse phenomenon of dissolution, for example, the adsorption of tryptophan by carbon black from aqueous urea solution reported in the previous paper,[12] which could be explained on the basis of the existing views on hydrophobic bonding. Conclusively, the concept regarding the enthalpy of mixing described in this paper seems applicable generally to explaining the phenomenon under various experimental conditions such as discussed above, and also seems to afford an intensive and useful means to an approach to an understanding of biopharmaceutical phenomena.